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Two temperature-sensitive mutations in the DNA binding subunit of *Eco*KI with differing properties

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Abstract

Two temperature-sensitive mutations in the *hsdS* gene, which encodes the DNA specificity subunit of the type IA restriction-modification system *Eco*KI, designated Sts1 (Ser^{340}Phe) and Sts2 (Ala^{204}Thr) had a different impact on restriction-modification functions in vitro and in vivo. The enzyme activities of the Sts1 mutant were temperature-sensitive in vitro and were reduced even at 30°C (permissive temperature). Gel retardation assays revealed that the Sts1 mutant had significantly decreased DNA binding, which was temperature-sensitive. In contrast the Sts2 mutant did not show differences from the wild-type enzyme even at 42°C. Unlike the HsdSts1 subunit, the HsdSts2 subunit was not able to compete with the wild-type subunit in assembly of the restriction enzyme in vivo, suggesting that the Sts2 mutation affects subunit assembly. Thus, it appears that these two mutations map two important regions in HsdS subunit responsible for DNA–protein and protein–protein interactions, respectively. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Temperature-sensitive mutant; DNA binding; Protein–protein interaction; Subunit assembly

1. Introduction

The type I restriction and modification (R-M) systems are grouped into four families (IA, IB, IC and ID) on the basis of gene order, amino acid conservation and enzymatic properties [1,2]. The restriction endonuclease *Eco*KI of *Escherichia coli* K12 is a member of the type IA family. It is a complex multifunctional enzyme composed of three different subunits (HsdR, HsdM and HsdS) which are encoded by the *hsd* genes. The *hsdR* gene is transcribed from its own promoter (P_{RES}), while the *hsdM* and *hsdS* genes are transcribed from a separate promoter (P_{MOD}) [2]. The subunit stoichiometry of the endonuclease is $R_2M_2S_1$. The HsdS and HsdM subunits can also form an independent DNA methyltransferase with a stoichiometry of M_2S_1, which is an intermediate in assembly of the endonuclease [3,4]. *Eco*KI recognises an asymmetric bipartite DNA sequence of AACnnnnnnGTGC, but cuts DNA at random sites distant to the recognition sequence as a consequence of ATP-dependent DNA translocation past the enzyme, which remains bound to the recognition site [5]

DNA specificity of the type I R-M enzymes is determined by the HsdS subunit [6]. The HsdS polypeptide contains two DNA target recognition domains (TRD) separated by a short region which is conserved within each type I family. There is another conserved region at the C-terminus of the polypeptide. Both conserved regions are thought to be involved in protein–protein interactions with HsdM and HsdR [7] while each TRD is responsible for recognising one half of the asymmetric recognition sequence [8,9]. Structure prediction methods suggest that all type I TRDs have a similar tertiary structure related to that of the TRD of H*ha* I methyltransferase, whose crystal structure has been determined [10].

We have investigated two point mutations in the *hsdS* gene of *Eco*KI showing a temperature-sensitive phenotype

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Abbreviations: AdoMet, *S*-adenosylmethionine; TRD, target recognition domain; ts, temperature-sensitive

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in vivo [11,12]. One of the mutations (Sts1) changes serine 340 to phenylalanine and displays a $r_{ts}m_{ts}$ phenotype [13]. The other mutation (Sts2) changes alanine 204 to threonine and shows a $r^{-}m_{ts}$ phenotype [11]. Sts1 is located in the distal TRD (214–368 aa) and Sts2 lies at the junction between the central conserved region and the distal TRD [10]. Interestingly, when the cellular level of all three Hsd subunits, including HsdSts1, was increased by cloning the *hsd* genes into a multi-copy plasmid, under control of their natural promoters, the bacteria showed the wild-type R-M phenotype. However, purified mutant endonuclease showed reduced restriction and modification activity in vitro [14]. In this paper we have analysed the DNA binding properties of both mutants, by means of a gel retardation assay, to determine if the observed phenotypes result from a defect in DNA binding. With the Sts1 mutant we indeed demonstrated that the mutant endonuclease has significantly reduced DNA binding in a temperature-sensitive manner when compared to that of the wild-type enzyme. This suggests that Ser^{340} of HsdS subunit may be important for DNA binding. The Sts2 mutant did not show significant differences from the wild-type in vitro. However, the in vivo subunit competition experiments suggested that the ts2 mutation may affect protein assembly at the non-permissive temperature.

2. Materials and methods

2.1. Microbiological techniques

Bacterial strains and plasmids used in this work are listed in Table 1. In vivo restriction and modification assays were carried out as described previously [6]. Antibiotics were used in the following concentrations: ampicillin, 100 mg ml^{-1}; chloramphenicol, 50 mg ml^{-1}.

2.2. Plasmid constructions

Plasmid pWU128 was constructed by ligating the *Hin*dIII fragment of pBg3 [15] carrying the *hsdR* and *hsdM* genes and the proximal part of the *hsdS* gene into the *Hin*dIII site of the *hsdS*ts2 gene in a derivative of plasmid pZH9 [11]. The *Hin*dIII site and the *hsdM* gene in plasmid pZH9 were removed by *Eco*RI-*Hpa*I and *Sal*I-*Bam*HI deletions respectively. The Sts2 mutation is located downstream of the *Hin*dIII site. Plasmid pWU20 was constructed by ligating the *Nae*I fragment of the *Eco*RI-*Hpa*I deletion derivative of pZH9, carrying the *hsdS*ts2 gene, with the *Nae*I fragment of plasmid pMSk64 [16] carrying the promoter region of lambda *cI857*.

2.3. Protein purification and analysis

The wild-type and mutant endonucleases were produced at 37°C in *E. coli* C3-6 harbouring recombinant plasmids pVMC3 (wild-type), pVM39 (Sts1) and pWU128 (Sts2) respectively and purified as described previously [14]. As judged by densitometric quantification of a SDS-PAGE of the final preparations, both mutants showed the same ratio of the subunits (HsdR, HsdM and HsdS) as the wild-type enzyme (data not shown).

2.4. DNA cleavage and ATPase assays

Restriction activity of the wild-type and mutant endonucleases was monitored as the cleavage of covalently closed plasmid DNA to linear DNA. 25 nM pDRM-2R DNA was pre-incubated with 50 nM enzyme in a buffer containing 50 mM Tris (pH 8), 25 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.2 mM *S*-adenosylmethionine (AdoMet) for 1 min at either 30°C or 42°C. The DNA cleavage reaction was afterwards initiated by addition of ATP to a final concentration of 10 mM. Aliquots were withdrawn and analysed on agarose gel. Following ethidium bromide staining, gels were photographed and photographic negatives were quantified by densitometry using Image Grabber2.1 and NIH Image 1.52 software as previously described [17]. ATPase activity was monitored by measuring the concentration of inorganic phosphate using a colorimetric assay described previously [17].

Table 1
Bacterial strains and plasmids

Strains	Characteristics	Reference
E. coli C122	prototroph, Δ*hsd*	British Culture Collection Strain No. 122
E. coli C3-6	*recA56* derivative of *E. coli* C122	[14]
E. coli C600	*thr, leu, thi*	[22]
Plasmids		
pMSk64	Ap^R, P_R-*hsdS*	[16]
pVM30	Ap^R, P_R-*hsdS* ts1	[12]
pWU20	Ap^R, P_R-*hsdS* ts2	this work
pVMC3	Ap^R, P_{RES}-*hsdR* P_{MOD}-*hsdMS*	[14]
pVM39	Ap^R, P_{RES}-*hsdR* P_{MOD}-*hsdMS* ts1	[14]
pWU128	Ap^R, P_{RES}-*hsdR* P_{MOD}-*hsdMS* ts2	this work
pDRM-2R	Ap^R, one *Eco*KI site	[17]

The lambda P_R promoter is regulated from the *cI857* repressor present on the *Bgl*II fragment used for the construction of pMSk64 [16].

2.5. Methylation assay

DNA methylation activity was assayed by measuring incorporation of tritiated methyl groups from [^{3}H]AdoMet to DNA. Hemimethylated DNA of the plasmid pDRM-2R was used as the DNA substrate, prepared by mixing equimolar amounts of in vivo *Eco*KI-modified (from *E. coli* C600) and unmodified (from *E. coli* C122) forms of the linearised plasmid as described [14]. Typically, 20 nM DNA was incubated with 40 nM enzyme in the same buffer as used in the DNA cleavage assay. The [^{3}H]AdoMet (Amersham) was present at a concentration of 1 μM.

2.6. Gel retardation assay

Complementary 30-mer oligonucleotides containing one *Eco*KI recognition site used in the assay were purchased HPLC-purified from Cruachem (top strand: 5′-d-CCGA-ATTC**AAC**CCCGGG**GTGC**AAGCTTGCC-3′).

The oligonucleotides were annealed and the duplex was labelled at the 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase. DNA binding reactions were performed in a volume of 10 μl in the presence of a buffer consisting of 50 mM Tris (pH 8.0), 25 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT and 10% (v/v) glycerol. 1 nM end-labelled DNA duplexes were incubated with the wild-type and mutant endonucleases over a range of concentrations at either 30°C or 42°C for 5 min. Unbound and bound DNA were separated on a 5% polyacrylamide non-denaturing TAE gel at 4°C at 100 V. After electrophoresis, gels were dried and subjected to autoradiography.

3. Results and discussion

3.1. DNA cleavage and ATPase activities

As a DNA substrate for assaying the in vitro restriction activities of the mutants plasmid pDRM-2R DNA was used [17]. This plasmid contains one recognition site for *Eco*KI. Fig. 1A compares the rate of DNA cleavage activ-

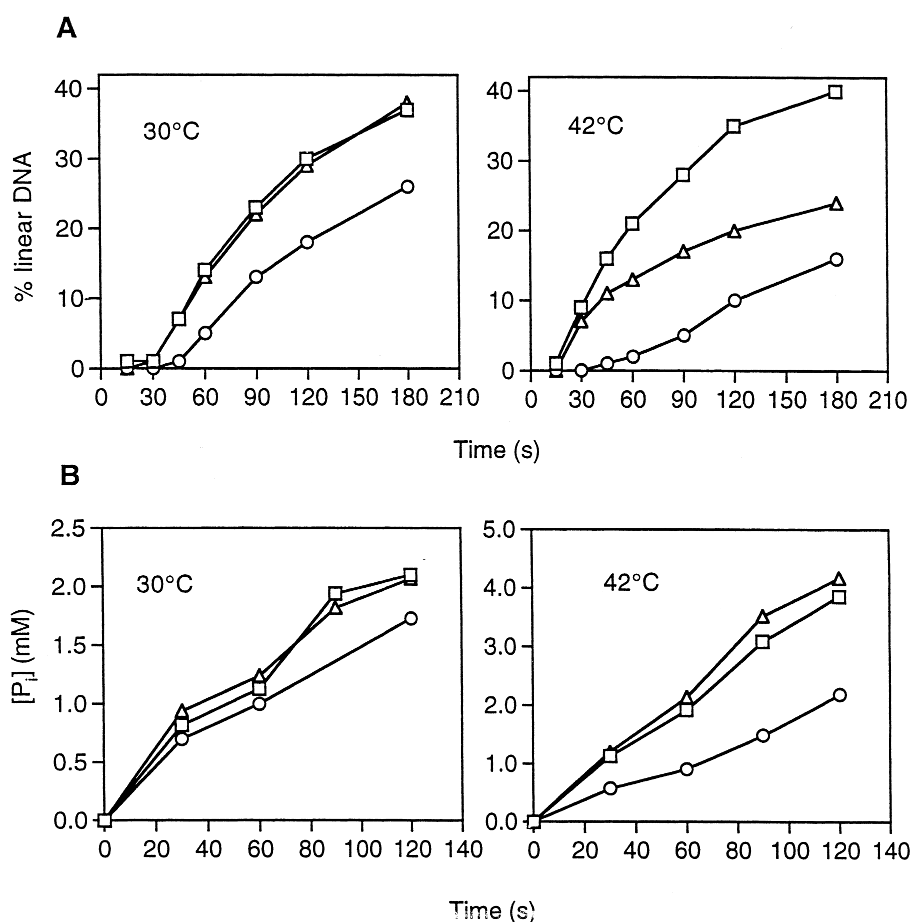

Fig. 1. Restriction and ATPase activities of the mutant *Eco*KI endonucleases. Restriction activity was monitored as a conversion of covalently closed DNA of plasmid pDRM-2R to linear DNA. 25 nM DNA was incubated with 50 nM enzyme at either 30°C or 42°C under conditions described in Section 2. Graphs in A are plots of relative concentration of linear DNA against time for incubations at 30°C and 42°C, respectively. ATPase activity was determined by measuring the concentration of inorganic phosphate (Pi) released by ATP hydrolysis. Graphs in B show a time course of ATPase activity of the wild-type and mutant enzymes at 30°C and 42°C, respectively. For all graphs, squares represent the wild-type, circles Sts1 and triangles Sts2 enzymes.

ities of the two mutants with the wild-type enzyme at 30°C and 42°C, respectively. Restriction activities of both mutants were found to be temperature-sensitive. However, the reduction in the restriction activity at 42°C was much more significant in the case of the Sts1 mutant than in the case of the Sts2 mutant. The Sts1 mutant had reduced restriction activity even at 30°C while restriction activity of Sts2 mutant at 30°C is the same as that of the wild-type enzyme.

ATPase activity was assayed in the same reaction aliquots that were used to measure the restriction activity. As can be seen in Fig. 1B, ATPase activity of the Sts1 mutant was temperature-sensitive and was reduced even at 30°C in comparison to the wild-type. ATPase activity of the Sts2 mutant did not show significant differences from the wild-type enzyme at either temperature.

3.2. *In vitro DNA methylation*

DNA methylation activity was measured as a transfer of tritiated methyl groups from [^{3}H]AdoMet to a hemimethylated pDRM-2R DNA. Kinetics of the methylation reaction at 30°C with 40 nM wild-type enzyme and the mutants is shown in Fig. 2A. As we observed previously [14], methylation activity of the Sts1 mutant was completely abolished under these conditions. The Sts2 mutant showed a slight reduction in methylation activity at 30°C. However, when the concentration of Sts1 in the reaction mixture was increased, a methylation activity was detected (Fig. 2B), which correlates with its modification activity detected in vivo [14]. Incubation at 42°C further reduced methylation activity of Sts1 while it did not have a significant effect on Sts2 (data not shown).

3.3. *DNA binding*

DNA binding of the wild-type and mutant enzymes was measured by gel retardation assays using a ^{32}P-radiolabeled 30-mer synthetic oligoduplex containing one *Eco*KI recognition site. An example of binding of the endonucleases to 1 nM oligoduplex DNA at various protein concentrations is shown in Fig. 3. The incubations were performed either at 30°C or at 42°C. Gel retardation assays revealed that the DNA binding of Sts1 was reduced at 30°C in comparison with the wild-type enzyme. The difference between wild-type and Sts1 mutant in DNA binding was even more significant at 42°C. The Sts2 mutant did not show any difference from wild-type at 30°C and only a small decrease in DNA binding at 42°C was observed. Since DNA binding is the first step in the enzyme reaction mechanism, the altered DNA binding properties of Sts1 are presumably the cause of the defect in restriction and modification activity of this mutant observed in vivo and in vitro. It should be noted that the extra DNA-protein complex appearing at low protein concentrations (Fig. 3) reflects dissociation of the $R_2M_2S_1$ endonuclease complex to the $R_1M_2S_1$ species and free HsdR subunit [18]. In addition, the faint band at the top of the gel at high protein concentrations may correspond to a higher molecular mass species, or non-specific binding. Thus the reduced DNA binding of the Sts1 mutant suggests that serine 340 of HsdS is important for DNA binding, correlating with its location within the distal TRD.

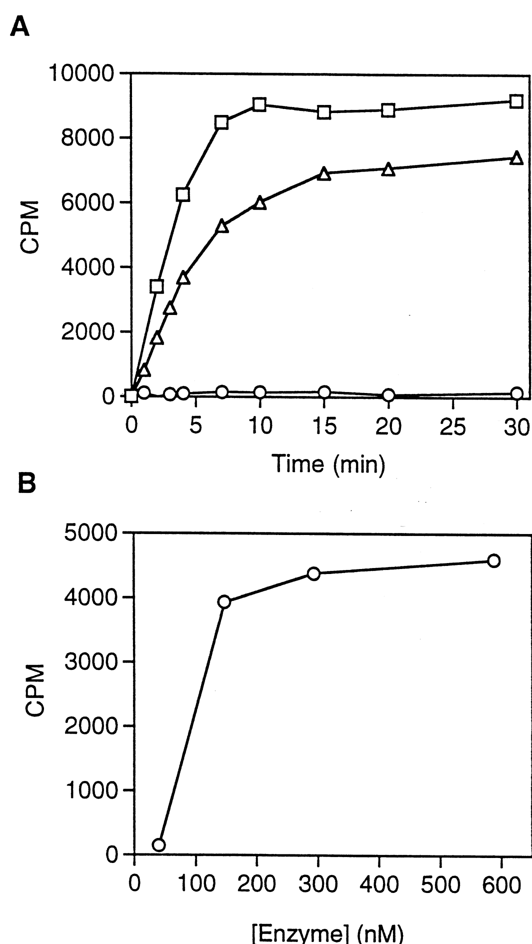

Fig. 2. In vitro DNA methylation by the mutant *Eco*KI endonucleases. The methylase activity was assayed by measuring of incorporation of tritiated methyl groups from [^{3}H]AdoMet to hemimethylated DNA as described in Section 2. The graph in A is a time course of methylation of 20 nM heteroduplex pDRM-2R DNA with 40 nM wild-type and mutant endonucleases at 30°C. The graph in B shows the influence of enzyme concentration on methylase activity of Sts1 endonuclease with 10 nM heteroduplex pDRM-2R DNA.

3.4. *Competition between mutant and wild-type HsdS subunits in enzyme assembly in vivo*

It was shown that over-production of HsdSts1 subunit in cells expressing the wild-type *Eco*KI R-M system abolished restriction and modification at the non-permissive temperature, indicating the trans-dominant nature of the Sts1 mutation [13]. The expression of dominance is found when the activity of multi-subunit complex is 'poisoned' by incorporation of a defective subunit [19]. One can con-

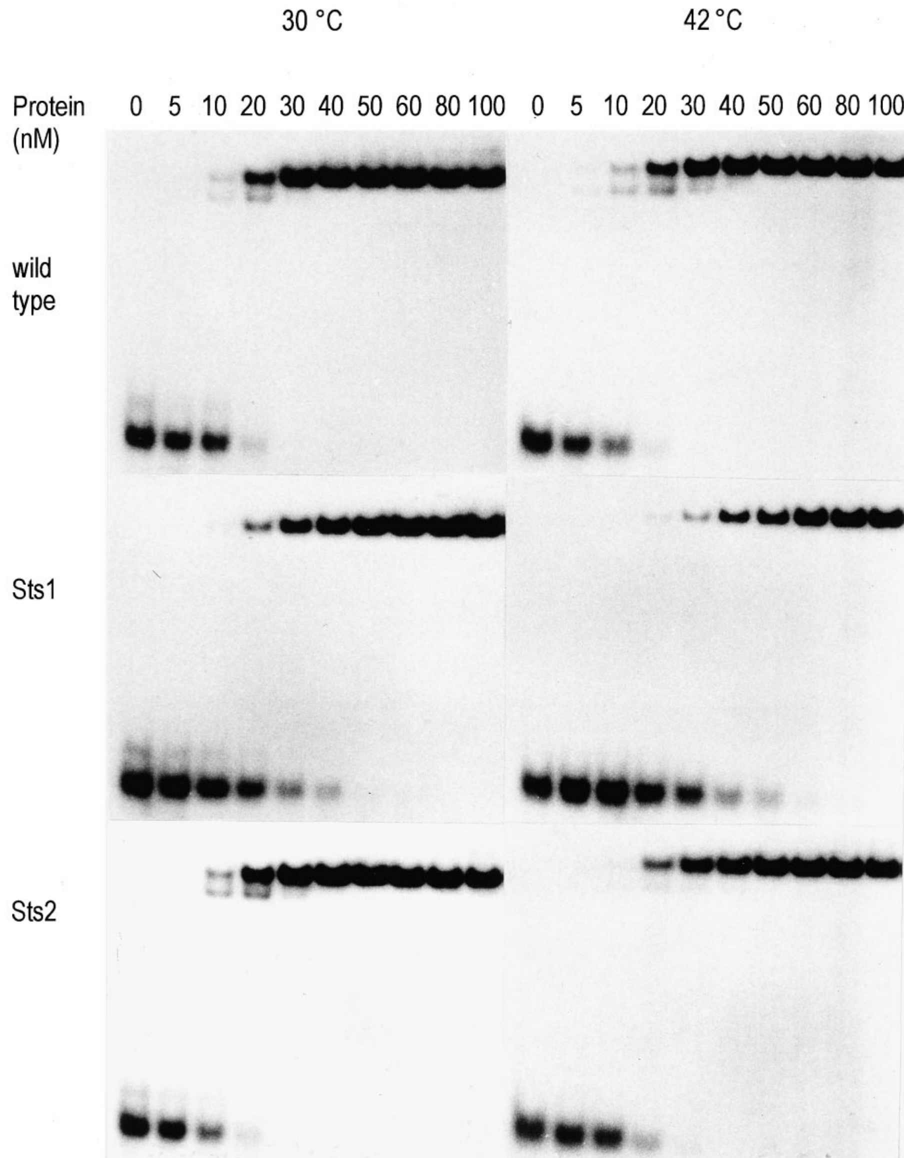

Fig. 3. Binding of the wild-type and mutant endonucleases to 30-mer DNA oligoduplex containing one *Eco*KI recognition site analysed by gel retardation assay. 1 nM ^{32}P-labelled DNA was incubated with increasing protein concentrations for 5 min at either 30°C or 42°C. In both cases, the protein was first pre-incubated without DNA for 1 min at the appropriate temperature. After the incubations the mixtures were immediately placed on ice as recommended in [23] and subsequently analysed on a native 5% TAE polyacrylamide gel as described in Section 2. Protein concentration and incubation temperatures are indicated.

clude from these data that the Sts1 mutation affects DNA binding of HsdS rather than protein–protein interactions with the other subunits. This was confirmed by in vitro analysis of DNA binding of the mutant enzyme described above (Fig. 3).

The purified Sts2 endonuclease did not show significant differences from the wild-type enzyme (Figs. 1–3), although the cells expressing the mutant R-M system displayed a r^-m_{ts} phenotype [11]. To investigate the possibility that the Sts2 mutation affects subunit assembly at the non-permissive temperature and thus abolishes the enzyme activity in vivo, we performed the same subunit competition assay as used with the Sts1 mutant. As seen in Fig. 4, over-production of HsdSts2 subunit from lambda P*R* in *E. coli* C600 (r^+m^+) had no effect on restriction activity at 42°C, unlike the observation of a trans-dominant effect with the Sts1 mutation. The negative trans-dominance is consistent with either a fully functional HsdS subunit, or a subunit incapable of protein–protein interaction [20]. Since the Sts2 mutation displays a ts phenotype under natural condition (chromosomal location of all *hsd* genes [11]) we favour the second alternative. We suggest that the mutant subunit is altered in its ability to make protein-protein interactions at the non-permissive temperature, which is in agreement with a previous proposal that the mutant methylase is unstable [11]. Moreover, we have recently shown that the equilibrium between the methylase (stoichiometry M_2S_1) and the M and M_1S_1 sub-assembly com-

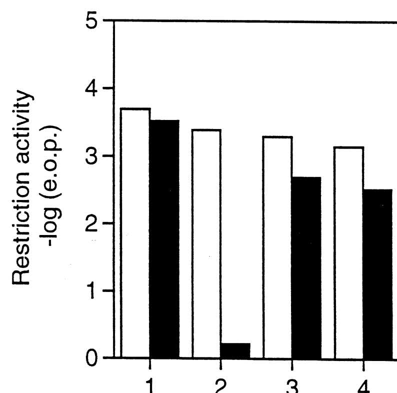

Fig. 4. Subunit competition assay showing effect of over-production of HsdSts1 and HsdSts2 subunits from the lambda PR promoter on restriction activity of *E. coli* C600 expressing the wild-type *Eco*KI R-M system from chromosomal *hsd* locus. Restriction activity is expressed as (−log) of efficiency of plating (e.o.p.) of bacteriophage lambda. White bars restriction at 30°C, black bars restriction at 42°C. Lane 1, control without plasmid; lane 2, HsdSts1 subunit over-produced from pVM30; lane 3, HsdSts2 from pWU20 and lane 4, wild-type HsdS from pMSk64.

ponents is shifted in favour of the partially assembled form in a temperature-dependent manner (unpublished results, D. Dryden, K. Firman, V. Zinkevich, and B. Hussey; for further discussion see [3]), confirming our conclusion that the Sts2 mutation affects subunit assembly at the non-permissive temperature.

The Sts2 mutation, $Ala^{204}Thr$, is located at the junction between the central conserved domain and TRD2 [10]. A similar location has been described for a non-classical mutation of the *Eco*R124I R-M system [21]. This mutant also appears to be altered in the subunit assembly pathway, indicating that this region of the HsdS subunit may be critically important for protein–protein interactions.

Acknowledgements

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